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PCT

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(54) Title: END-COMPLEMENTARY POLYMERASE REACTION

(57) Abstract

The present invention is directed to a process for amplifying and detecting any target nucleic acid sequence contained in a nucleic acid or mixture thereof and for assembling large polynucleotides from component polynucleotides, each involving generating concatemers formed by PCR amplification of overlapping fragments.

END-COMPLEMENTARY POLYMERASE REACTION

TECHNICAL FIELD

5   The present invention relates generally to the field
of recombinant DNA technology and, more particularly, to
improved methods for producing amplified heterogeneous
populations of polynucleotides from limited quantities of DNA
or RNA or other nucleic acids. The invention provides
10   compositions and methods for a chain reaction amplification of
a target polynucleotide species using a thermostable
polymerase or other suitable polynucleotide polymerase
compatible with the method.

15   BACKGROUND
          Selective amplification of polynucleotides
represents a major research goal of molecular biology, with
particular importance in diagnostic and forensic applications,
as well as for general manipulations of genetic materials and
20   laboratory reagents.
          The polymerase chain reaction (PCR) is a method by
which a specific polynucleotide sequence can be amplified _in
vitro_. PCR is an extremely powerful technique for amplifying
specific polynucleotide sequences, including genomic DNA,
25   single-stranded cDNA, and mRNA among others. As described in
U.S. Patent Nos. 4,683,202, 4,683,195, and 4,800,159 (which
are incorporated herein by reference), PCR typically comprises
treating separate complementary strands of a target nucleic
acid with two oligonucleotide primers to form complementary
30   primer extension products on both strands that act as
templates for synthesizing copies of the desired nucleic acid
sequences. By repeating the separation and synthesis steps in
an automated system, essentially exponential duplication of
the target sequences can be achieved.
35   A number of variations of the basic PCR methodology
have been described. U.S. Patent No. 5,066,584 discloses a
method wherein single stranded DNA can be generated by the
polymerase chain reaction using two oligonucleotide primers,

Triglia et al. (1988) <u>Nucl.Acids Res.</u> <u>16:</u> 8186,
describe an approach which requires the inversion of the
sequence of interest by circularization and re-opening at a
site distinct from the one of interest, and is called

5   "inverted PCR." A fragment is first created in which two
unknown sequences flank on either side a region of known DNA
sequence. The fragment is then circularized and cleaved with
an unique restriction endonuclease which only cuts within the
known DNA sequence creating a new fragment containing all of

10 the DNA of the original fragment but which is then inverted
with regions of known sequence flanking the region of unknown
sequence. This fragment is then utilized as a PCR substrate
to amplify the unknown sequence.

Vallette et al. (1989) <u>Nucl.Acids Res.</u> <u>17:</u> 723

15 disclose using PCR in a specific approach which involves using
a supercoiled plasmid DNA as a template for PCR and a primer
bearing a mutated sequence which is incorporated into the
amplified product. Using this method, DNA sequences may be
inserted only at the 5'-end of the DNA molecule which one

20 wishes to alter. Mole et al. (1989) <u>Nucl.Acids Res.</u> <u>17:</u>
3319, used PCR to create deletions within existing expression
plasmids. However, PCR was performed around the entire
plasmid (containing the fragment to be deleted) from primers
whose 5'-ends defined the region to be deleted. Self-ligation

25 of the PCR product recircularized the plasmid.

U.S. Patent No. 5,279,952 discloses a method for
using PCR to generate mutations (e.g., deletions) and chimeric
genes by forming head-to-tail concatemers of a known starting
sequence and employing at least two PCR primers to amplify a

30 DNA segment which is altered as compared to the known starting
sequence.

Jones and Howard (1990) <u>BioTechniques</u> <u>8:</u> 178, report
a site-specific mutagenesis method using PCR, termed
recombinant circle PCR (RCPCR). In RCPCR, separate PCR

35 amplifications (typically two) of a known polynucleotide
generate products that, when combined, denatured, and
annealed, form double-stranded DNA with discrete, cohesive

thermostable polymerase (e.g., <u>Thermus aquaticus</u> (Taq)
polymerase or <u>Thermococcus litoralis</u> (Vent™, New England
Biolabs) polymerase or TthI polymerase (Perkin-Elmer).
Although the method can be practiced using PCR, in some
5   embodiments either a single primer species or no primer
whatsoever is required, and hence PCR is not a necessary
component of the general method.

In one embodiment, a target polynucleotide is
contacted with a "bivalent primer" typically comprising an
10   oligonucleotide having two regions of complementarity to the
target polynucleotide: (1) a first portion which is in the 5'
portion of the primer and which is substantially complementary
to a sequence in the 3' portion of the sequence to be
amplified (target sequence) in the target polynucleotide, and
15   (2) a second portion which is in the 3' portion of the primer
and which is substantially complementary to a sequence in the
5' portion of the sequence to be amplified (target sequence)
in the target polynucleotide. The contacting is performed
under conditions suitable for hybridization of the bivalent
20   primer to the target polynucleotide, most often following
thermal denaturation of the target polynucleotide if it is
initially present as a double-stranded form. The target
polynucleotide may be substantially homogeneous or may be
present in a mixture of polynucleotide species (e.g., in a
25   genome, biological sample, or mixture of synthetic
polynucleotides). Subsequent or concomitant with the
contacting of the bivalent primer to the target
polynucleotide, a polynucleotide polymerase, such as a
thermostable DNA polymerase, catalyzes, under suitable
30   reaction conditions, polynucleotide synthesis (chain
elongation) primed from the 3'-hydroxyl of the annealed
bivalent primer to form a strand complementary to the target
sequence, thereby forming a nascent complementary strand.
Following completion of the nascent complementary strand
35   spanning the target sequence, the target polynucleotide and
the nascent strand are denatured, typically by elevation of
temperature, and allowed to reanneal, typically by reduction
of temperature, with another molecule of the bivalent primer

a concatemeric single strand emanating from the rolling circle
intermediate. Most often, such rolling circle intermediates
will form under dilute conditions more favorable to
intramolecular cyclization of overlapped strands. Once a

5   rolling circle intermediate is established, the template need
not be denatured in order to continue amplification of the
target sequence as in conventional PCR, thus avoiding the
necessity of multiple thermal cycles of PCR to denature
template (and the resultant time loss needed for heating and

10 cooling). Often, however, the template is repeatedly
denatured, annealed, and extended with polymerase in the
presence of ribonucleotide or deoxyribonucleotides under
suitable reaction conditions.

        Furthermore, whether the method generates a rolling

15 circle intermediate or linear concatemers, an advantage of the
method is that it requires a substantially reduced amount of
primer (bivalent primer) as compared to conventional PCR,
since following the initial cycle(s) an increasing percentage
of the priming of nascent strand synthesis is primed from 3'-

20 hydroxyl groups of the amplified strands, rather than from the
oligonucleotide primer(s). In the case of a rolling circle
intermediate, theoretically only a single bivalent primer
molecule is necessary to generate the rolling circle which
then can produce multiple concatenated copies by rolling

25 circle-style polymerase catalysis using a polymerase capable
of strand displacement of the lagging edge of the nascent
strand as replication proceeds around the cyclized template.

        In an embodiment, a product polynucleotide is
assembled from a plurality of component polynucleotides by

30 formation of overlapped strands of alternating polarity and
having substantially complementary termini (see, Fig. 3).
This method employs a series of overlapping substantially
complementary termini to determine the linear order of
component sequences in the final product. Concomitant with or

35 subsequent to formation of the overlapped strands of the
component polynucleotides in a reaction, a polynucleotide
polymerase (e.g., a thermostable DNA polymerase) under
suitable reaction conditions catalyzes strand elongation from

into the product polynucleotide(s) during amplification
cycles. The method can be used to produce a library of
sequence-variant product polynucleotides, if desired.

In an embodiment of the invention, very long
5   distance PCR is provided, wherein PCR or other suitable
amplification method is used to generate, in a single reaction
or in parallel reactions which are subsequently pooled, a set
of overlapping large DNA fragments which can be denatured and
annealed to form very large (e.g., greater than 25 to 50
10 kilobases) DNA structures composed of overlapped single
strands of DNA having alternating polarity with each
overlapped joint providing an extendable 3'-hydroxyl group for
forming phosphodiester bonds catalyzed by a polynucleotide
polymerase in the presence of free ribonucleotide or
15 deoxyribonucleotides. Typically, the method comprises forming
at least three overlapping polynucleotides, wherein the 3'
terminus of a first single-stranded polynucleotide is
substantially complementary to the 3' terminus of a second
single-stranded polynucleotide of the opposite polarity, and
20 wherein the 5' terminus of said second single-stranded
polynucleotide is substantially complementary to the 3'
terminus of a third single-stranded polynucleotide having
polarity identical to said first single-stranded
polynucleotide, thereby generating an overlapped structure
25 capable of chain elongation by a suitable polymerase to
generate a double-stranded product spanning the three initial
overlapped polynucleotides. With such a method,
polynucleotides of 50 kb to 100 kb or more can be generated by
a facile amplification method capable of generating
30 amplification products much longer than is possible with
conventional long-range PCR methods. The method can comprise
parallel processing PCR reactions, wherein a plurality of
primer sets are employed in a single reaction or multiple
reactions which are subsequently pooled, each primer sets
35 priming the PCR amplification of a polynucleotide sequence
which comprises terminal sequences which are complementary to
terminal sequences in at least one other amplification product
produced by a different primer set, thus generating a set of

nuclease S1, bleomycin, ionizing radiation, or the like or by other suitable cleaving means.

A further understanding of the nature and advantages of the invention will become apparent by reference to the
5   remaining portions of the specification and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1.   Schematic of bivalent primer and concatemer formation in end-complementary PCR.
10   Fig. 2   Schematic depiction of cyclized intermediates and rolling circle amplification.

Fig. 3   Schematic of overlapping fragment PCR for construction and amplification of larger products from component polynucleotides.
15   Fig. 4   Schematic of multiple "alleles" with overlapping fragment PCR.

Fig. 5A-E shows oligonucleotides used to construct the 2.7 kb circular plasmid p182SfiI by end-complementary polymerase reaction.
20   Fig. 6   Schematic of plasmid construction by overlapping fragment PCR as performed in Experimental Examples.

Fig. 7   Agarose gel electrophoresis of products generated during construction of the 2.7 kb circular plasmid
25   p182SfiI by end-complementary polymerase reaction. The circled letters refer to aliquots removed from various amplification reactions: (A) is the mixture of oligonucleotides without polymerase, (B) is the product of the first set of amplification cycles, (C) is the product of the
30   second set of amplification cycles, (D) is the final product.

Fig. 8   Schematic for end-complementary polymerase reaction (ECPR) in conjunction with parallel-processing PCR to amplify very large polynucleotides, such as those larger than can be amplified reliably by conventional PCR using only a
35   single primer set.

Figs. 9A-9C   Schematic of continuous circular multiplex amplification methodology exemplifying amplification of two unlinked polynucleotides, double-stranded ABC/A'B'C'

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univalent primers are F' and A. In some embodiments, X and X'
and/or Y and Y' can be omitted. Fig. 10A shows the initial
(starting) conditions with a plurality of polynucleotide
species (shown for example as two double-stranded molecules)
5   to be amplified by continuous circular multiplex
amplification. The second step, "Anneal with Primers", shows
the structural features of the bivalent and univalent primers
and their mode of annealing to single-stranded polynucleotide
species, shown as denatured double-stranded complementary
10 polynucleotides. The third step, "Extend and Melt", shows the
amplification products after a cycle of extension primed by
the bivalent and univalent primers; the amplification products
are denatured for a subsequent round(s) of amplification.
Fig. 10B shows the possible modes of reannealing of the
15 amplification products generated from the first round of
amplification. Fig. 10C shows the amplification products
which can result from the second round (or subsequent rounds)
of amplification; each of the product molecules comprises
copies of the initially unlinked polynucleotide sequences in
20 equimolar ratios.

        Figs. 11A-11C Schematic of continuous circular
multiplex amplification methodology exemplifying amplification
of two possibly unlinked polynucleotides embedded in distinct
locations in a genome or pool of DNA molecules. Fig. 11A
25 shows the initial (starting) conditions with a plurality of
polynucleotide sequences (shown for example as two double-
stranded sequences embedded in discrete genomic locations) to
be amplified by continuous circular multiplex amplification.
First, the genomic sequences are amplified using a low
30 concentration of conventional amplification primers (shown as
PCR primers; C',F',A, and D) as indicated under "Anneal #1".
The concentration of conventional primers, initial copy
number, and number of amplification cycles is such that
primers for rapidly extending fragments are consumed and
35 slowly extending sequences are allowed to amplify. Fig. 11B
shows that bivalent primers (FT3'XT7A and CYD) are used in
subsequent rounds of amplification. In this example, one of
the bivalent primers (FT3'XT7A) comprises the sequences for

methods and materials are described. For purposes of the
present invention, the following terms are defined below.

Definitions

5   Unless specified otherwise, the conventional
notation used herein portrays polynucleotides as follows: the
lefthand end of single-stranded polynucleotide sequences is
the 5' end; the lefthand direction of double-stranded
polynucleotide sequences is referred to as the 5' direction.
10 The direction of 5' to 3' addition of nascent RNA transcripts
is referred to as the transcription direction; sequence
regions on the DNA strand having the same sequence as the RNA
and which are 5' to the 5' end of the RNA transcript are
referred to as "upstream sequences"; sequence regions on the
15 DNA strand having the same sequence as the RNA and which are
3' to the 3' end of the coding RNA transcript are referred to
as "downstream sequences".

          As used herein, the term "polynucleotide" refers to
a polymer composed of a multiplicity of nucleotide units
20 (ribonucleotide or deoxyribonucleotide or related structural
variants) linked via phosphodiester bonds. A polynucleotide
can be of substantially any length, typically from about 10
nucleotides to about 1×10^9 nucleotides or larger. As used
herein, an "oligonucleotide" is defined as a polynucleotide of
25 from 6 to 100 nucleotides in length. Thus, an oligonucleotide
is a subset of polynucleotides.

          The term "naturally-occurring" as used herein as
applied to an object refers to the fact that an object can be
found in nature. For example, a polypeptide or polynucleotide
30 sequence that is present in an organism (including viruses)
that can be isolated from a source in nature and which has not
been intentionally modified by man in the laboratory is
naturally-occurring. Generally, the term naturally-occurring
refers to an object as present in a non-pathological
35 (undiseased) individual, such as would be typical for the
species.

          The term "corresponds to" is used herein to mean
that a polynucleotide sequence is homologous (i.e., is

the local homology algorithm of Smith and Waterman (1981) _Adv._
Appl. Math. 2: 482, by the homology alignment algorithm of
Needleman and Wunsch (1970) _J. Mol. Biol._ 48: 443, by the
search for similarity method of Pearson and Lipman (1988)
5   _Proc. Natl. Acad. Sci. (U.S.A.)_ 85: 2444, by computerized
implementations of these algorithms (GAP, BESTFIT, FASTA, and
TFASTA in the Wisconsin Genetics Software Package Release 7.0,
Genetics Computer Group, 575 Science Dr., Madison, WI), or by
inspection, and the best alignment (i.e., resulting in the
10 highest percentage of homology over the comparison window)
generated by the various methods is selected.

The term "sequence identity" means that two
polynucleotide sequences are identical (i.e., on a nucleotide-
by-nucleotide basis) over the window of comparison. The term
15 "percentage of sequence identity" is calculated by comparing
two optimally aligned sequences over the window of comparison,
determining the number of positions at which the identical
nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both
sequences to yield the number of matched positions, dividing
20 the number of matched positions by the total number of
positions in the window of comparison (i.e., the window size),
and multiplying the result by 100 to yield the percentage of
sequence identity. The terms "substantial identity" as used
herein denotes a characteristic of a polynucleotide sequence,
25 wherein the polynucleotide comprises a sequence that has at
least 80 percent sequence identity, preferably at least 85
percent identity and often 90 to 95 percent sequence identity,
more usually at least 99 percent sequence identity as compared
to a reference sequence over a comparison window of at least
30 20 nucleotide positions, frequently over a window of at least
25-50 nucleotides, wherein the percentage of sequence identity
is calculated by comparing the reference sequence to the
polynucleotide sequence which may include deletions or
additions which total 20 percent or less of the reference
35 sequence over the window of comparison. The reference
sequence may be a subset of a larger sequence.

The primers herein are selected to be substantially
complementary to the different strands of each specific

substantially complementary to a sequence in the 5' and 3'
portions of the sequence to be amplified (target sequence) are
each at least 12 to 15 nucleotides in length, often 18 to 20
nucleotides in length, and are preferably 100 percent

5   identical to the complement of the annealing portion of the
target sequence. Often, bivalent primers of the invention are
oligonucleotides.

       The term "primer" as used herein refers to an
oligonucleotide whether occurring naturally as in a purified

10 restriction digest or produced synthetically, which is capable
of acting as a point of initiation of synthesis when placed
under conditions in which synthesis of a primer extension
product which is complementary to a nucleic acid strand is
induced, i.e., in the presence of nucleotides and an agent for

15 polymerization such as DNA polymerase and at a suitable
temperature and pH. The primer is preferably single-stranded
for maximum efficiency in amplification, but may alternatively
be double stranded. If double stranded, the primer is first
treated to separate its strands before being used to prepare

20 extension products. Preferably, the primer is an
oligodeoxyribonucleotide. The primer must be sufficiently long
to prime the synthesis of extension products in the presence
of the agent for polymerization. The exact lengths of the
primers will depend on many factors, including temperature and

25 source of primers. For example, depending on the complexity
of the target sequence, the oligonucleotide primer typically
contains 15-25 or more nucleotides, although it may contain
fewer nucleotides. Short primer molecules generally require
cooler temperatures to form sufficiently stable hybrid

30 complexes with template. In some embodiments, the primers can
be large polynucleotides, such as from about 200 nucleotides
to several kilobases or more.

       As used herein, "suitable reaction conditions" are
those conditions suitable for conducting PCR amplification

35 using conventional reagents. Such conditions are known or
readily established by those of skill in the art, and can be
exemplified by the reaction conditions used in U.S. Patents
4,683,202, 4,683,195, and 4,800,159, which are incorporated

fluorescent labels (e.g., FITC, rhodamine, lanthanide
phosphors), enzymatic labels (e.g., horseradish peroxidase, β-
galactosidase, luciferase, alkaline phosphatase), biotinyl
groups, and the like. In some embodiments, labels are
5   attached by spacer arms of various lengths to reduce potential
steric hindrance.

      As used herein, "substantially pure" means an object
species is the predominant species present (i.e., on a molar
basis it is more abundant than any other individual
10 macromolecular species in the composition), and preferably a
substantially purified fraction is a composition wherein the
object species comprises at least about 50 percent (on a molar
basis) of all macromolecular species present. Generally, a
substantially pure composition will comprise more than about
15 80 to 90 percent of all macromolecular species present in the
composition. Most preferably, the object species is purified
to essential homogeneity (contaminant species cannot be
detected in the composition by conventional detection methods)
wherein the composition consists essentially of a single
20 macromolecular species. Solvent species, small molecules
(<500 Daltons), and elemental ion species are not considered
macromolecular species.

      The term "recombinant" used herein refers to
macromolecules produced by recombinant DNA techniques wherein
25 the gene coding for a polypeptide is cloned by known
recombinant DNA technology. For example, an amplified or
assembled product polynucleotide may be inserted into a
suitable DNA vector, such as a bacterial plasmid, and the
plasmid used to transform a suitable host. The gene is then
30 expressed in the host to produce the recombinant protein. The
transformed host may be prokaryotic or eukaryotic, including
mammalian, yeast, Aspergillus and insect cells. One preferred
embodiment employs bacterial cells as the host.
Alternatively, the product polynucleotide may serve a non-
35 coding function (e.g., promoter, origin of replication,
ribosome-binding site, etc.).

      Generally, the nomenclature used hereafter and many
of the laboratory procedures in cell culture, molecular

thermostable polymerase (e.g., Thermus aquaticus (Taq)
polymerase or Thermococcus litoralis (Vent™) polymerase. In
an embodiment, the method is performed using PCR, typically
with multiple cycles of heat denaturation and DNA synthesis.
5   However, there are several variations of the basic method of
end-complementary polymerase reaction which are exemplified
hereinbelow and which shall be evident to the skilled artisan
in view of the present specification. Some variations do not
require primers and/or sequential cycles of thermal
10   denaturation.

          In embodiments where the product size increases with
the number of denaturation, annealing, and extension cycles
(e.g., as the mean length of concatemers increase), it is
typically advantageous to increase the denaturation
15   temperature, and optionally increase the reannealing time, for
subsequent cycles. Such conditions are readily optimized by
the practitioner using pilot reactions to establish a
calibration curve for any particular embodiment.

20   Single-Primer Amplification
          A target polynucleotide is contacted with a bivalent
primer typically comprising an oligonucleotide having two
regions of complementarity to the target polynucleotide: (1) a
first portion which is in the 5' portion of the primer and
25   which is substantially complementary to a sequence in the 3'
portion of the sequence to be amplified (target sequence) in
the target polynucleotide, and (2) a second portion which is
in the 3' portion of the primer and which is substantially
complementary to a sequence in the 5' portion of the sequence
30   to be amplified (target sequence) in the target
polynucleotide. The contacting is performed under conditions
suitable for hybridization of the bivalent primer to the
target polynucleotide for polymerase-mediated chain
elongation, most often following thermal denaturation of the
35   target polynucleotide if it is initially present as a double-
stranded form.

          The first portion of the bivalent primer which is in
the 5' portion of the primer and which is substantially

length and is frequently less than 50 to 500 nucleotides in
length. The first portion of the bivalent primer is
substantially identical to the complement of a sequence at the
5' end of the target sequence, however there may be additional
5   terminal nucleotides of the first portion of the bivalent
primer which are substantially non-identical to a target
sequence or its complement. Such terminal nucleotides must be
substantially non-interfering so that their presence does not
significantly inhibit the capability of the bivalent primer to
10 selectively anneal to the target sequence and initiate chain
elongation under suitable reaction conditions in the presence
of polymerase. Although the first portion of the bivalent
primer is substantially identical to the complement of a
sequence at the 5' end of the target sequence, it need not be
15 exactly identical; often a sequence identity of at least 80
percent is sufficient, typically at least 90 percent sequence
identity is present, and preferably at least 95 percent or 100
percent sequence identity is present. In some embodiments,
sequence identity of less than 80 percent is practicable, but
20 the amount of sequence identity and length of overlap for the
joints is determined by the discretion of the practitioner.
        The amount of sequence identity necessary for any
given application will vary depending on several factors
including: (1) complexity of the population of polynucleotides
25 in which the target polynucleotide(s) is/are present, (2)
temperature and ionic strength, (3) sequence composition of
the target sequence, (4) length of sequence identity, and (5)
size of the primer. Practitioners will select bivalent
primers having a first portion with sufficient sequence
30 identity and length to serve as selective amplification
primers which specifically hybridize to the desired target
polynucleotide(s). Specific hybridization is the formation of
hybrids between a primer polynucleotide and a target
polynucleotide, wherein the primer polynucleotide
35 preferentially hybridizes to the target DNA such that, for
example, at least one discrete band can be identified on a gel
of amplification products obtained from amplification of
genomic DNA prepared from eukaryotic cells that contain (or

complementary strand of a target polynucleotide or an
amplified copy thereof. The denatured nascent strand species
following the first elongation cycle will contain a copy of
the target sequence and has a terminal repeat of its 5'-
5   terminal sequence at the 3' terminus, resulting from the
bivalent primer, and wherein the terminal repeat is of
sufficient length to support annealing under PCR conditions to
an overlapping complementary strand in a head-to-tail
arrangement (see, Fig. 1). Following reannealing, the
10   described polymerase elongation/denaturation/reannealing cycle
is repeated from 1 to about 100 times as desired, resulting in
formation of amplified product which comprises head-to-tail
concatemers of the target sequence. The concatemers typically
increase in length as the number of amplification cycles
15   increase and as the amount of bivalent primer decreases.
Following amplification forming concatameric head-to-tail ·
repeats of the target sequence, the concatemer(s) can
optionally be resolved, such as (1) by cleaving with a
restriction endonuclease which cuts within (or at the termini
20   of) the concatemeric unit(s), (2) by homologous recombination
between concatemer units to form covalently closed circles, or
(3) by cleavage with a restriction endonuclease followed by
ligation with DNA ligase to form covalently closed circles
and/or by direct transformation into host cells for in vivo
25   ligation.

         By this method, a single primer (bivalent primer) is
used to amplify a target polynucleotide sequence having a
predetermined 5' terminal sequence and a predetermined 3'
terminal sequence. The predetermined 5' terminal sequence and
30   a predetermined 3' terminal sequence may be contained
internally within a larger polynucleotide; hence the use of
the term "terminal" refers only to their terminality within
the target sequence, not necessarily the complete target
polynucleotide which may be a superset of the target sequence.

35

Rolling Circle PCR Amplification

         Often, a target polynucleotide sequence which is
amplified by the present method will form amplification

alternating polarity and having substantially complementary
termini (see, Fig. 3). This method employs a series of
overlapping substantially complementary termini to determine
the linear order of component sequences in the final product.

5   Concomitant with or subsequent to formation of the overlapped
strands of the component polynucleotides in a reaction, a
polynucleotide polymerase (e.g., a thermostable DNA
polymerase) under suitable reaction conditions catalyzes
strand elongation from the 3'-hydroxyl portions of the

10   overlapped (annealed) joints, filling in the portion between
joints and processively displacing or processively degrading
exonucleolytically the 5' termini of downstream component
strands of the same polarity as the nascent strand elongates.
After a cycle of chain elongation forming substantially

15   double-stranded polynucleotides, the reaction conditions are
altered (typically by increasing the temperature) to effect
denaturation of the double-stranded polynucleotides, followed
by altering the reaction conditions to permit reannealing of
complementary strands or portions thereof (i.e., overlapping

20   termini) to form molecules having overlapped termini (joints),
and chain elongation by a polynucleotide polymerase under
suitable reaction conditions catalyzes strand elongation from
the 3'-hydroxyl portions of the overlapped (annealed) joints,
as in the first cycle. One to about 100 cycles of

25   denaturation/annealing/polymerization can be performed to
generate a product comprising the component polynucleotide
sequences covalently linked in linear order according to the
order of the overlapping joints. In this embodiment, a
product polynucleotide can be constructed from a plurality of

30   smaller component polynucleotides (typically oligonucleotides)
and enables assembly of a variety of products with alternate
substitutable polynucleotide components at a given position
serving as structural "alleles" (see, Fig. 4). The component
polynucleotides are often provided in single-strand form, but

35   may initially be present in double-strand form and be
denatured (typically by elevated temperature) for the assembly
of the product by PCR amplification. Substantially any type
of product polynucleotide can be assembled in this way,

31

number of bases at both ends of the sequence be known in
sufficient detail so that a bivalent primers can be prepared
which will hybridize to the desired sequence and at relative
positions along the sequence such that an extension product
5   initially synthesized from the bivalent primer, when it is
separated from its template (complement), can anneal with a
stand of the opposite polarity to form an overlapped joint of
a head-to-tail concatemer and serve as a template for
extension of the 3'-hydroxyl from each overlapped joint. The
10 greater the knowledge about the bases at both ends of the
sequence, the greater can be the specificity of the primer for
the target nucleic acid sequence, and thus the greater the
efficiency of the process. It will be understood that the
word bivalent primer as used hereinafter may refer to more
15 than one bivalent primer, particularly in the case where there
is some ambiguity in the information regarding the terminal
sequence(s) of the fragment to be amplified. For instance, in
the case where a nucleic acid sequence is inferred from
protein sequence information a collection of primers
20 containing sequences representing all possible codon
variations based on degeneracy of the genetic code will be
used for each strand.

The polynucleotide primers may be prepared using any
suitable method, such as, for example, the phosphotriester and
25 phosphodiester methods, or automated embodiments thereof. In
one such automated embodiment diethylphosphoramidites are used
as starting materials and may be synthesized as described by
Beaucage et al. (1981) _Tetrahedron Letters_ __22:__ 1859. One
method for synthesizing oligonucleotides on a modified solid
30 support is described in U.S. Pat. No. 4,458,066. It is also
possible to use a primer which has been isolated from a
biological source (such as a restriction endonuclease digest
or the like.

The specific nucleic acid sequence is produced by
35 using the target polynucleotide containing that sequence as a
template. If the target polynucleotide contains two strands,
it is necessary to separate the strands of the nucleic acid
before it can be used as the template, either as a separate

about 45° C. The agent for polymerization may be any compound
or system which will function to accomplish the synthesis of
primer extension products, including enzymes. Suitable enzymes
for this purpose include, for example, E. coli DNA polymerase

5   I, Klenow fragment of E. coli DNA polymerase I, T4 DNA
polymerase, other available DNA polymerases, reverse
transcriptase, and other enzymes, including heat-stable
enzymes, which will facilitate combination of the nucleotides
in the proper manner to form the primer extension products

10   which are complementary to each nucleic acid strand.
Generally, the synthesis will be initiated at the 3' end of
the primer and proceed in the 5' direction along the
template strand, until synthesis terminates, producing
molecules of different lengths.

15   The newly synthesized strand and its complementary
nucleic acid strand form a double-stranded molecule which is
used in the succeeding steps of the process. In the next step,
the strands of the double-stranded molecule are separated
using any of the procedures described above to provide

20   single-stranded molecules.
            The steps of strand separation and extension product
synthesis can be repeated as often as needed to produce the
desired quantity of the specific nucleic acid sequence. The
amount of the specific nucleic acid sequence produced will

25   accumulate in an exponential fashion and the average size of
the product will also increase as the length of the
concatemers increases with each cycle.
            The method herein may also be used to enable
detection and/or characterization of specific nucleic acid

30   sequences associated with infectious diseases, genetic
disorders or cellular disorders such as cancer, e.g.,
oncogenes. Amplification is useful when the amount of nucleic
acid available for analysis is very small, as, for example, in
the prenatal diagnosis of sickle cell anemia using DNA

35   obtained from fetal cells.

Continuous Multiplex Amplification

generate increasingly longer amplification products which
contain the initial unlinked (or distantly linked) sequences
in equal amounts. After completion of amplification, several
options can be pursued; the amplification product(s) can be

5 used directly, the X and/or Y sequences can contain
restriction sites (preferably unique sites) to allow digestion
with the restrictino enzyme and, if desired, separation and/or
purification of the two (or more) originally unlinked
sequences. Alternatively, or in combination, transcription

10 promoters (e.g., T3 and T7) can be included in X and/or Y
sequences to facilitate transcription of the amplified
sequences. Figs. 10A-10C show a linear format of the
continuous multiplex amplification method. Figs. 11A-11C show
an embodiment of circular continuous multiplex amplification

15 wherein bivalent primers contain T3 and T7 promoters and the
functional promoter sequences are thereby introduced into the
amplification product(s).

        The following examples are given to illustrate the
invention, but are not to be limiting thereof.

20

EXPERIMENTAL EXAMPLES
        The following examples are offered by way of example
and not by way of limitation. Variations and alternate
embodiments will be apparent to those of skill in the art.

25

Example 1. LacZ alpha gene reassembly
        This example shows that small fragments having
overlapping regions of homology can be amplified and
reassembled by PCR amplification methods in the absence of any

30 primer.

1)   Substrate preparation
        The substrate for the reassembly reaction was the dsDNA
polymerase chain reaction ("PCR") product of the wild-type

35 LacZ alpha gene from pUC18. (Gene Bank No. XO2514) The primer
sequences were 5'AAAGCGTCGATTTTTGTGAT3' (SEQ ID NO:1) and
5'ATGGGGTTCCGCGCACATTT3' (SEQ ID NO:2). The free primers were
removed from the PCR product by Wizard PCR prep (Promega,

30 seconds, 50°C for 30 seconds and 72°C for 30 seconds), a single product of the correct size was obtained.

5)   Cloning and analysis

5   The PCR product from step 4 above was digested with the terminal restriction enzymes BamHI and EcoO109 and gel purified as described above in step 2. The reassembled fragments were ligated into pUC18 digested with BamHI and EcoO109. E. coli were transformed with the ligation mixture
10 under standard conditions as recommended by the manufacturer (Stratagene, San Diego CA) and plated on agar plates having 100 μg/ml ampicillin, 0.004% X-gal and 2mM IPTG. The resulting colonies having the HinDIII-NheI fragment which is diagnostic for the ++ recombinant were identified because they
15 appeared blue.
        This Example illustrates that a 1.0 kb sequence carrying the LacZ alpha gene can be digested into 10-70 bp fragments, and that these gel purified 10-70 bp fragments can be reassembled to a single product of the correct size, such that
20 84% (N=377) of the resulting colonies are LacZ+ (versus 94% without shuffling). This principal finding is extended substantially in the present invention to assemble component polynucleotides into product polynucleotides, and the component polynucleotides are not limited to randomly digested
25 fragments of a naturally-occurring gene sequence.
        The DNA encoding the LacZ gene from the resulting LacZ- colonies was sequenced with a sequencing kit (United States Biochemical Co., Cleveland OH) according to the manufacturer's instructions and the genes were found to have point mutations
30 due to the reassembly process (Table 1). 11/12 types of substitutions were found, and no frameshifts.

A collection of 132 component oligonucleotides, each 40 bases
in length, as well as one 56-mer and one 47-mer (<u>see</u>, Fig. 5A-
E) were synthesized and used to assemble the plasmid by end-
complementary polymerase reaction (ECPR) employing the

5   overlapping ends of the oligonucleotides. This collection of
component polynucleotides collectively encode the plasmid
p182SfiI. The plus strand and the minus strand were each
initially directed by oligonucleotides 40 nucleotides long
which, upon assembly, overlapped by 20 nucleotides (Fig. 6).

10 The oligonucleotides were synthesized and 5'-phosphorylated
simultaneously on a 96-well parallel-array DNA synthesizer
using standard phosphoramidite chemistry. After cleavage from
the solid support and deprotection, the dried down
oligonucleotides were resuspended in distilled water and used

15 without further purification.

        The oligonucleotides were diluted to a final
concentration (all oligos combined) of 1μM (14 ng/μl) in 20 μl
of GeneAMP XL PCR Mix (Perkin-Elmer, Branchburg, NJ; 0.2mM
each dNTP, 2.2 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0,

20 0.1% Triton X-100). An aliquot of the reaction mix (A) was
electrophoresed on an agarose gel (Fig. 7). The reaction was
started with 5 μl of a 50:1 (v/v) mixture of Taq polymerase
(Promega, Madison, WI) and Pfu polymerase (Stratagene, La
Jolla, CA) such that 1 unit of Taq and 0.02 unit of Pfu

25 polymerase were added. The PCR program consisted of the
following program: 40°C for 2 minutes, 72°C for 10 seconds,
then 40 cycles of (94°C for 15 seconds, 40°C for 30 seconds,
and 72°C for [10 seconds + 1 second/cycle]). An aliquot of
the resulting reaction product (B) was electrophoresed on an

30 agarose gel (Fig. 7), the remainder was then diluted 3x with
XL PCR Mix and enzyme and amplified with the following
program: 25 cycles of (94°C for 15 seconds, 40°C for 30
seconds, and 72°C for [45 seconds + 1 second/cycle]). An
aliquot of the resulting reaction product (C) was

35 electrophoresed on an agarose gel (Fig. 7) and the remainder
then diluted 3x with XL PCR Mix and enzyme and amplified with
the following program: 20 cycles of (94°C for 15 seconds, 40°C
for 30 seconds, and 72°C for [70 seconds + 1 second/cycle]).

Example 3. Antibody Germline Assembly from Oligos With Rolling
Circle Concatemeric Amplification

A scFv antibody with germline sequences (V_H251 and
$V_{IA}25$) was constructed from 19 oligonucleotides by cyclized
5   assembly. The oligos were at 2-20 ng per μl in PCR Mix. the
program was 20 cycles of (94°C for 15s, 48°C for 30s, 72°C for
30+1s/cycle). The size of the product of this reaction was
200-500 bp. The PCR product was diluted 4-fold in PCR Mix and
PCR was run for 24 cycles of (94°C for 15s, 55°C for 30s, 72°C
10 for 30+8s/cycle), followed by one additional 3-fold dilution
and 20 cycles of (94°C for 15s, 55°C for 30s, 72°C for
30+8s/cycle). The product was >50 kilobases, and after
digestion with SfiI and NotI resulted in a single DNA fragment
of the correct size.
15   Fig. 8 shows a schematic for end-complementary
polymerase reaction (ECPR) in conjunction with parallel-
processing PCR to amplify very large polynucleotides, such as
those larger than can be amplified reliably by conventional
PCR using only a single primer set.
20

Example 4. Plasmid Assembly With Rolling Circle Concatemeric
Amplification

pGJ103 is a 5.5 kilobase plasmid containing an
25 intact ars operon (Ji and Silver (1992) Proc. Natl. Acad. Sci.
(USA) 89: 9474).
In one example, pGJ103 was digested with DNAseI into
random 100-400 bp fragments which were reassembled by circular
shuffling in PCR Mix with a program of 50 cycles of (94°C for
30 15s, 68°C for 30s+8s/cycle), using three different
concentrations of fragments. Each reassembly yielded a
product of >50 kb which was digested with BamHI to yield a
single band of the correct (predicted) size, which was
ligated, transformed into E. coli, and preplated on increasing
35 levels of arsenate to select for up-mutants.

Cells and plasmids. Plasmid pGJ103 is pUC19 derivative
containing the 2.5 kb arsenic resistance operon from S. aureus

Cell growth was quantitated by resuspending the cells and
measuring the OD600nm.

Arsenate detoxification assay. The ability of E. coli
5   constructs to detoxify arsenate was measured by intact cell
arsenate reduction assay using radioactive 73AsO43-as
substrate and separation of arsenate and arsenite of by thin
layer chromatography, followed by quantitation in an Ambis
radioactive counter.
10

DNA sequencing. The sequence of the entire operon after
selection was determined by dideoxy DNA sequencing using
fluorescent terminating substrate and an ABI sequencer.

15 Results and discussion. The wild type plasmid pGJ103 ars
operon confers on E. coli strain TG1 resistance to up to 4 mM
arsenate when grown on LB plates at 37°C for 24 hrs.
Selection round one, which was plated on 2, 4, 8, 16 and 32 mM
arsenate, yielded about 2,000 colonies growing at 16 mM
20 arsenate. Selection round two was plated 16, 32, 64 and 128
mM arsenate and yielded about 4,000 colonies growing at 64 mM
arsenate. Round three was plated at 64, 128 and 256 mM
arsenate and yielded about 1,500 colonies at 128 mM arsenate,
and round 4 was plated on 128, 256 and 512 mM arsenate.
25 Colonies were harvested from the plates with 256 mM arsenate
and replated on 200, 300 and 400 mM arsenate. Single colonies
from plates with 400 mM arsenate were grown in liquid culture
with 400 mM arsenate and frozen at -70°C, and used for further
characterization. Resistance levels were increased by DNA
30 shuffling to arsenate (as selected) and also to arsenite and
antimony salts (Fig. 12), which are the two toxic oxyanions to
which resistance requires the ArsB membrane transporter but
not the ArsC arsenate reductase enzyme. In this growth
experiment, done with the pool from three cycles of DNA
35 shuffling (which retained good growth in LB broth), not only
was growth clear about 100 mM AsO43-, but increased resistance
to arsenite (AsO2-) and antimony (SbO3+) was clearly=20 shown.
These results require mutational effects beyond those possibly

the pI258 operon, and not from the E. coli K-12 chromosomal
operon.

Chromosomal shuffling. Because the cells recovered from 128
5   mM arsenate did not contain plasmid DNA, the shuffling for
round 4 was performed on the PCR product which was obtained
from the chromosomal DNA of the cells selected in round 3.
This PCR product was combined with a 10-fold lower molar
amount of the plasmid DNA obtained from round 2 cells, and the
10 mixture was fragmented, shuffled and selected as for earlier
rounds.

Cloning and characterization of the integrated operon. The
conventional PCR product which was obtained from the
15 chromosomal DNA of cells grown at 400 mM arsenate was cloned
into the polylinker site of pUC19. This construct was similar
to pGJ103 except that it lacked the 200 bp inverted homologous
DNA flanking the arsenate operon. Cells containing this
plasmid were resistant only up to about 10 mM arsenate. The
20 reason for this loss of arsenate resistance level is not
known. The DNA sequence of this cloned chromosomal operon
showed thirteen base changes relative to the original
sequence. The arsR gene contained two silent mutations (T389C
and T429C. The arsB gene contained ten base changes, and one
25 base change occurred in the non-coding area past the end of
the arsC gene (G2469C). Of the ten base changes in arsB,
three resulted in amino acid alterations: base T1281C change
resulted in amino acid change L232S, base T1317C change
resulted in amino acid change F244S, and base T1853C change
30 resulted in amino acid change Y423H, all three involving a
change toward a more hydrophilic residue via a T to C
transition. The seven silent mutations were T961G, A976G,
T1267C, A1402G, T1730C, T1819C and T1844C.

35 Arsenate reductase activity. The activity of arsenate
reductase by whole mutant cells after the third cycle was
increased about 50-fold (Fig. 13) to the wild type initial
strain with plasmid pGJ103. This increase in whole cell

47

CLAIMS:

1.   A method for amplifying a target polynucleotide, comprising:

5   contacting under conditions suitable for PCR, a target polynucleotide with a bivalent primer which comprises two portions of complementarity to the target polynucleotide: (1) a first portion which is in the 5' portion of the primer and which is substantially complementary to a sequence in the

10   3' portion of the sequence to be amplified (target sequence) in the target polynucleotide, and (2) a second portion which is in the 3' portion of the primer and which is substantially complementary to a sequence in the 5' portion of the sequence to be amplified (target sequence) in the target

15   polynucleotide;

           catalyzing under suitable reaction conditions for PCR, polynucleotide synthesis primed from the 3'-hydroxyl of the annealed bivalent primer to form a strand complementary to the target sequence, thereby forming a nascent complementary

20   strand;

           denaturing the target polynucleotide and the nascent strand and allowing reannealing with another molecule of the bivalent primer species or with a complementary strand of a target polynucleotide or an amplified copy thereof; and

25   repeating an elongation/denaturation/reannealing cycle from 1 to about 100 times as desired, resulting in formation of amplified product which comprises head-to-tail concatemers of the target sequence.

30   2.   The method of claim 1, comprising the further step of cleaving said concatemers with a restriction endonuclease which cuts within each concatemeric unit to form a population of polynucleotides each consisting of an amplified target sequence.

35

           3.   The method of claim 2, comprising the further step of ligating the population of polynucleotides each

· 49

conditions permitting reannealing of complementary strands or
portions thereof to form concatemers having overlapped joints,
and permitting chain elongation by a polynucleotide polymerase
from the 3'-hydroxyl portions of the overlapped joints; and

5   repeating said second step from 1 to 100 times to
generate a double-stranded concatemer of a desired size.

          7.   The method of claim 6, wherein the component
polynucleotides are in single-strand form.

10

          8.   The method of claim 6, wherein the polymerase
is Taq polymerase, TthI polymerase or Vent polymerase.

          9.   The method of claim 6, wherein the step of
15 denaturation comprises elevating the temperature of the
reaction to at least 94°C.

          10.   A kit comprising a bivalent primer and
instructions for performing the method of claim 1.

20

          11.   A method for producing a polynucleotide by
overlap assembly of parallel polymerase chain reaction
amplifications, comprising:
          forming at least three overlapping polynucleotides,
25 wherein the 3' terminus of a first single-stranded
polynucleotide is substantially complementary to the 3'
terminus of a third single-stranded polynucleotide of the
opposite polarity, and wherein the 5' terminus of said second
single-stranded polynucleotide is substantially complementary
30 to the 3' terminus of a third single-stranded polynucleotide
having polarity identical to said first single-stranded
polynucleotide, thereby generating an overlapped
polynucleotide set capable of chain elongation by a suitable
polymerase to generate a double-stranded product spanning the
35 three initial overlapped polynucleotides;
          synthesizing complementary strand polynucleotide
sequences catalyzed by a DNA polymerase to form a double-
stranded product spanning the overlapped polynucleotide set.

comprising at least one prokaryotic or bacteriophage promoter
sequence.

FIGURE 1A

A method to amplify single stranded DNA
fragments
using a single primer.

① Initial DNA

A' 5'   3' B'

Initial Primer

B A

2. Annealing

B A
3' ⬚ 5'
5' ⬚
8' 3'

5' A'

2. Extension

A   B A
3' ⬚   ⬚ 5'
5' ⬚   ⬚ 3'
A'   B A'
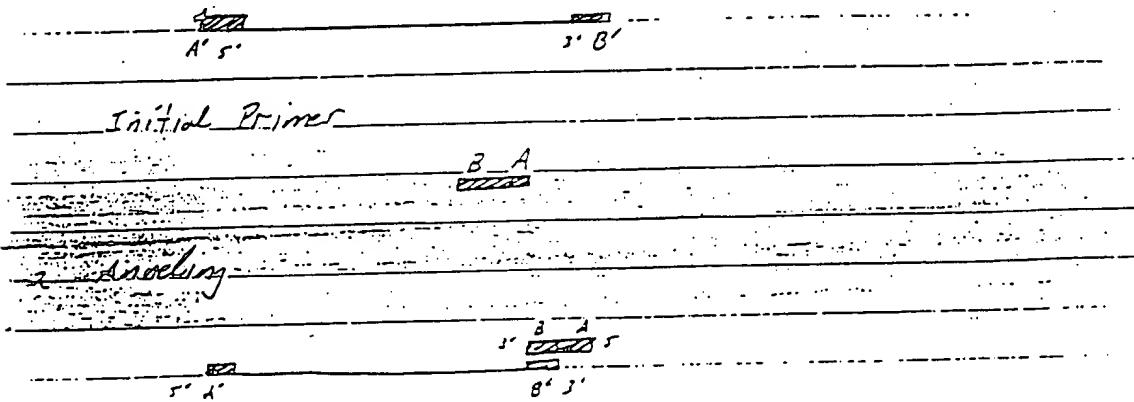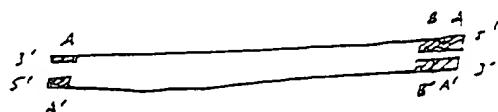
4. melt + reanneal to get # possible hybrids
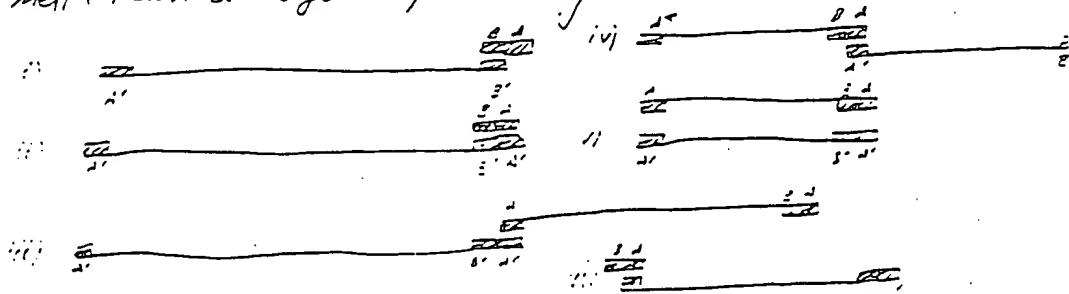
FIGURE 1B

Extend

    only hybrids i, ii, iii extend.

5. melt.
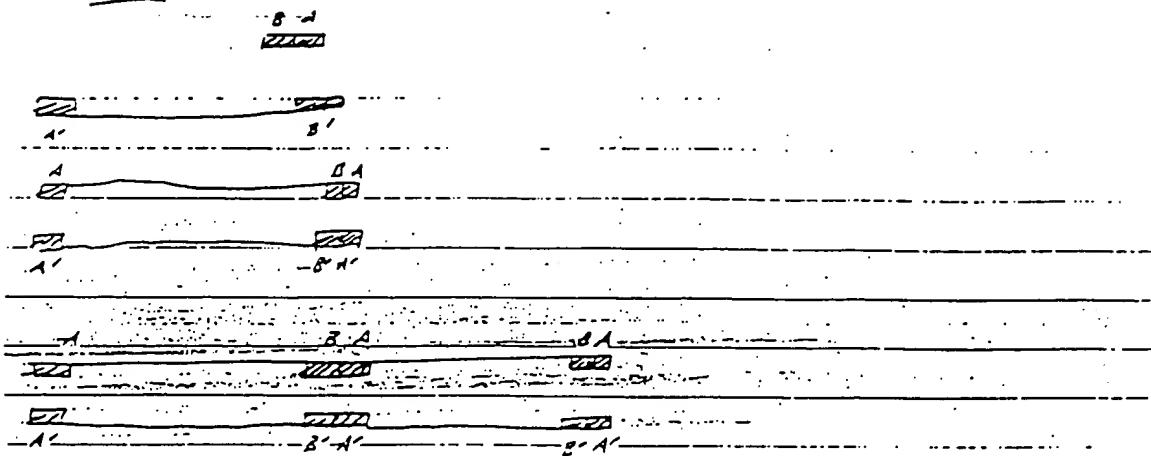
- Reannealing gives the following possible extendible hybrids

    i, ii, iii from step 4.
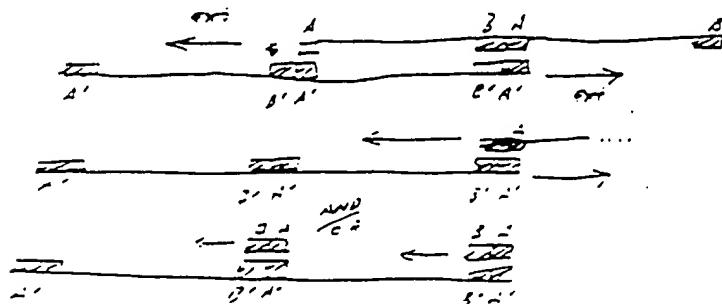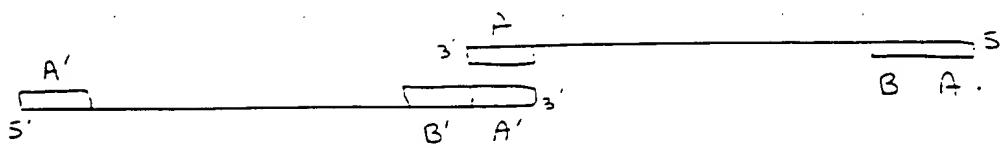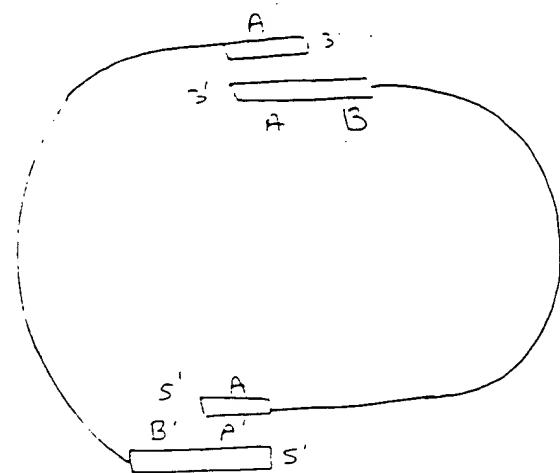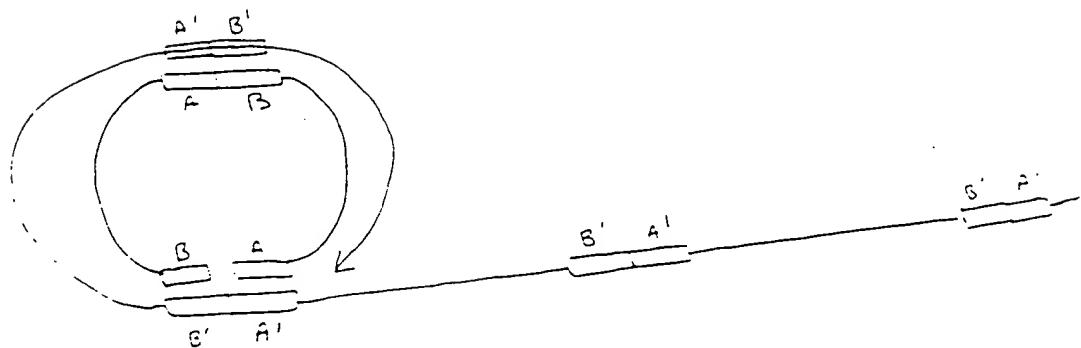
and.
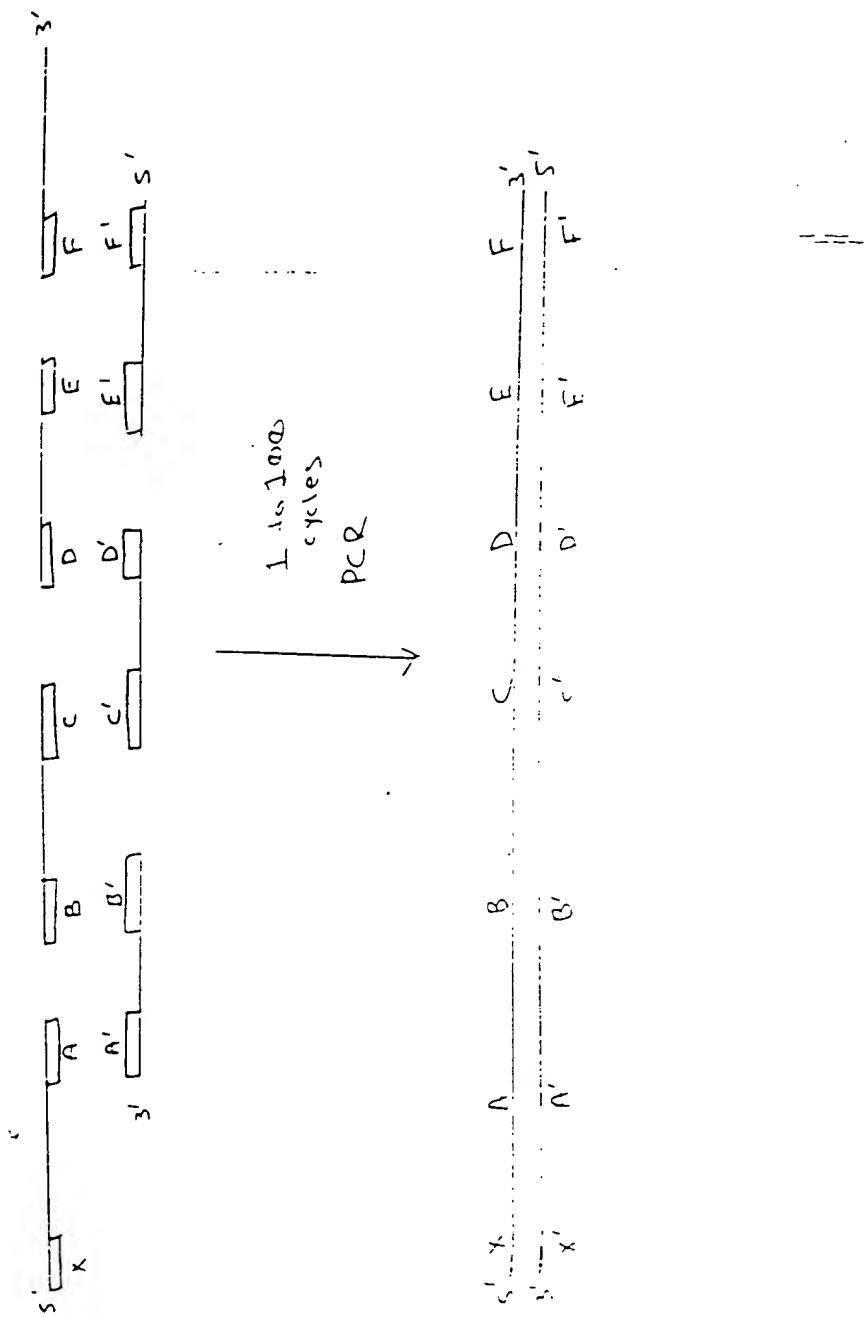
FIGURE 2

FIGURE 3
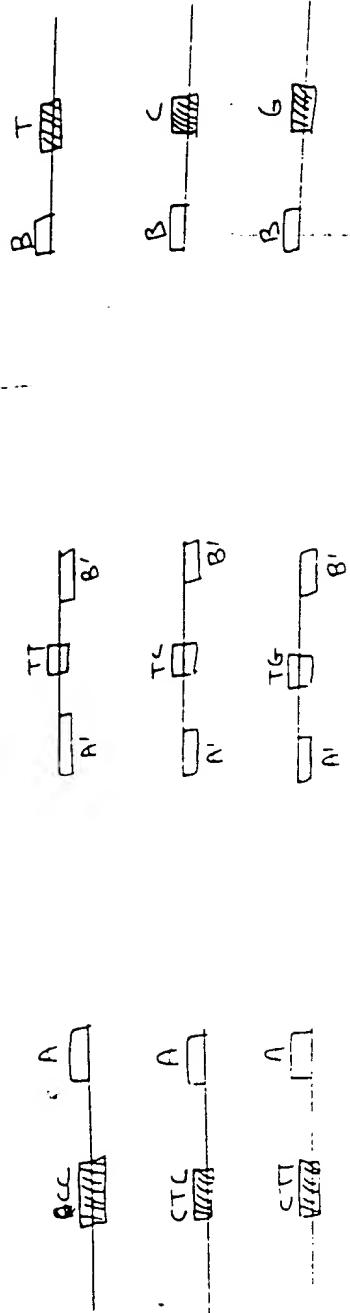
FIGURE 4

R1	CTC ACG TTA AGG GAT TTT GGT CAT GAG ATT ATC AAA AAG G
R2	ATC TTC ACC TAG ATC CTT TTA AAT TAA AAA TGA AGT TTT A
R3	AAT CAA TCT AAA GTA TAT ATG AGG CCT GAC AGG CCG GTC T
R4	GAC AGT TAC CAA TGC TTA ATC AGT GAG GCA GCT ATC TCA G
R5	CGA TCT GTC TAT TTC GTT CAT CCA TGG TTG CCT GAC TCC C
R6	CGT CGT GTA GAT AAC TAC GAT ACG GGA GGG CTT ACC ATC T
R7	GGC CCC AGT GCT GCA ATG ATA CCG CGA GAC CCA CGC TCA C
R8	CGG CTC CAG ATT TAT CAG CAA TAA ACC AGC CAG CTG GAA G
R9	GCC GGA GCG CAG AAG TGG TCC TGC AAC TTT ATC CGC CTC C
R10	ATC CAG TCT ATT AAT TGT TGC CGG GAA GCT AGA GTA AGT A
R11	GTT CGC CAG TTA ATA GTT TGC GCA ACG TTG TTG CCA TGG C
R12	TAC AGG CAT CGT GGT GTC ACG CTC GTC GTT TGG AAT GGC T
R13	TCA TTC AGC TCC GGT TCC CAA CGA TCA AGG CGA GTT ACA T
R14	GAT CCC CCA TGT TGT GCA AAA AAG CGG TTA GCT CCT TCG G
R15	TCC TCC GAT CGT TGT CAG AAG TAA GTT GGC TGC AGT GTT A
R16	TCA CTC ATG GTT ATG GCA GCA CTG CAT AAT TCT CTT ACT G
R17	TCA TGC CAT CCG TAA GAT GCT TTT CTG TGA CTG GTG AGT A
R18	CTC AAC CAA GTC ATT CTG AGA ATA GTG TAT GCG GCG ACC G
R19	AGT GCT CTT GCC CGG CGT CAA TAC GGG ATA ATA CCG CGC C
R20	CAC ATA GCA GAA CTT TAA AAG TGC TCA TCA TTG GAA AAC G
R21	TTC TTC GGG GCG AAA ACT CTC AAG GAT CTT ACC GCT GTT G
R22	AGA TCC AGT TCG ATG TAA CCC ACT CGT GCA CCC AAC TGA T
R23	CTT CAG CAT CTT TTA CTT TCA CCA GCG TTT CTG GGT GAG C
R24	AAA AAC AGG AAG GCA AAA TGC CGC AAA AAA GGG AAT AAG G
R25	GCG ACA CGG AAA TGT TGA ATA CTC ATA CTC TTC CTT TTT C
R26	AAT ATT ATT GAA GCA TTT ATC AGT GTT ATT GTC TCA TGA G
R27	CGG ATA CAT ATT TGA ATG TAT TTA GCC CAT GGT GGC CAA A
R28	AAT AAA CAA ATA GGG GTT CCG CGC ACA TTT CCC CGA AAA G

```
1   TTC TTA GAC GTC AGG TGG CAC TTT TCG CGG AAA TGT CCG C

2   GGA ACC CCT ATT TGT TTA TTT TTG GCC ACC ATG GCC TAA A

3   TAC ATT CAA ATA TGT ATC CGC TCA TGA GAC AAT AAC CCT G

4   ATA AAT GCT TCA ATA ATA TTG AAA AAG GAA GAG TAT GAG T

5   ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG G

6   CAT TTT GCC TTC CTG TTT TTG CTC ACC CAG AAA CGC TGG T

7   GAA AGT AAA AGA TGC TGA AGA TCA GTT GGG TGC ACG AGT G

8   GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT G

9   AGA GTT TTC GCC CCG AAG AAC GTT TTC CAA TGA TGA GCA C

10  TTT TAA AGT TCT GCT ATG TGG CGC GGT ATT ATC CCG TAT T

11  GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT T

12  CTC AGA ATG ACT TGG TTG AGT ACT CAC CAG TCA CAG AAA A

13  GCA TCT TAC GGA TGG CAT GAC AGT AAG AGA ATT ATG CAG T

14  GCT GCC ATA ACC ATG AGT GAT AAC ACT GCA GCC AAC TTA C

15  TTC TGA CAA CGA TCG GAG GAC CGA AGG AGC TAA CCG CTT T

16  TTT GCA CAA CAT GGG GGA TCA TGT AAC TCG CCT TGA TCG T

17  TGG GAA CCG GAG CTG AAT GAA GCC ATT CCA AAC GAC GAG C

18  GTG ACA CCA CGA TGC CTG TAG CCA TGG CAA CAA CGT TGC G

19  CAA ACT ATT AAC TGG CGA ACT ACT TAC TCT AGC TTC CCG G

20  CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT GCA G

21  GAC CAC TTC TGC GCT CGG CCC TTC CAG CTG GCT GGT TTA T

22  TGC TGA TAA ATC TGG AGC CGG TGA GCG TGG GTC TCG CGG T

23  ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT A

24  TCG TAG TTA TCT ACA CGA CGG GGA GTC AGG CAA CCA TGG A

25  TGA ACG AAA TAG ACA GAT CGC TGA GAT AGG TGC CTC ACT G

26  ATT AAG CAT TGG TAA CTG TCA GAC CGG CCT GTC AGG CCT C

27  ATA TAT ACT TTA GAT TGA TTT AAA ACT TCA TTT TTA ATT T

28  AAA AGG ATC TAG GTG AAG ATC CTT TTT GAT AAT CTC ATG A
```

(58F)
GGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCA
(59F)
CATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCC
(60F)
GCACCGATCGCCCTTCCCAACAGTTGCGTAGCCTGAATGG
(61F)
CGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTG
(62F)
TGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAA
(63F)
TCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCC
(64F)
GCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTC
(65F)
CCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGA
(66F)
GCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACG
(30R)
CGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGA
(31R)
AAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCT
(32R)
TGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGG
(33R)
GCGCGTCAGCGGGTGTTGGCGGGTCTCGGGGCTGGCTTAA
(34R)
CTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCAT
(35R)
ATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAT
(36R)
ACCGCATCAGGCGCCATTCGCCATTCAGGCTACGCAACTG
(37R)
TTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGC
(38R)
CAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTT
(39R)
GGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAAC
(40R)
GACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCT
(41R)
AGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGG
(42R)
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAA
(43R)
TTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGC
(44R)
CTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
(45R)
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGT
(46R)
GCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGG
(47R)
CGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACT
(48R)
GACTCGCTGCGCTCGGTCGTTCGGCTGCGCCGAGCGGTAT
(49R)
CAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC

FIG. 5C

(50R)
AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG
(51R)
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGT
(52R)
TTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA
(53R)
TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTA
(54R)
TAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
(55R)
GCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTC
(56R)
CGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGC
(57R)
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCT
(58R)
CCAAGCTGGGCTGTGTGCACGAACCCCCCCGTTCAGCCCGA
(59R)
CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAAC
(60R)
CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
(61R)
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC
(62R)
AGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGA
(63R)
AGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTA
(64R)
CCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA
(65R)
AACCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAG
(66R)
CAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTT
(67R)
TGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA

(67F)
CGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA

(29R)
TGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTAT

(29F)
CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTC
(30F)
AGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCT
(31F)
TTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAAC
(32F)
CACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCT
(33F)
ACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCG
(34F)
CAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAG
(35F)
GCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT
(36F)
CGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGC
(37F)
GATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGT
(38F)
TACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTC
(39F)
GTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA
(40F)
CTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGC
(41F)
TTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGG
(42F)
CAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGG
(43F)
GGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCC
(44F)
ACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGG
(45F)
GGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTT
(46F)
TTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGT
(47F)
TCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTAT
(48F)
TACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGA
(49F)
ACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG
(50F)
AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCC
(51F)
GATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGG
(52F)
AAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGC
(53F)
TCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCC
(54F)
GGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTT
(55F)
CACACAGGAAACAGCTATGACCATGATTACGAATTCGAGC
(56F)
TCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATG
(57F)
CAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTG

134 40-mers
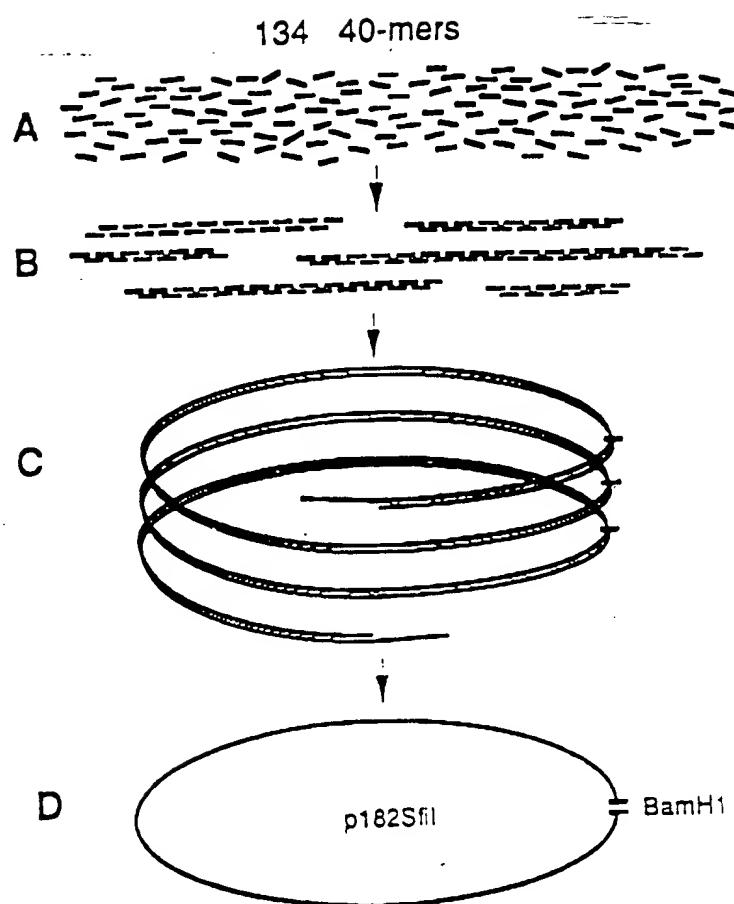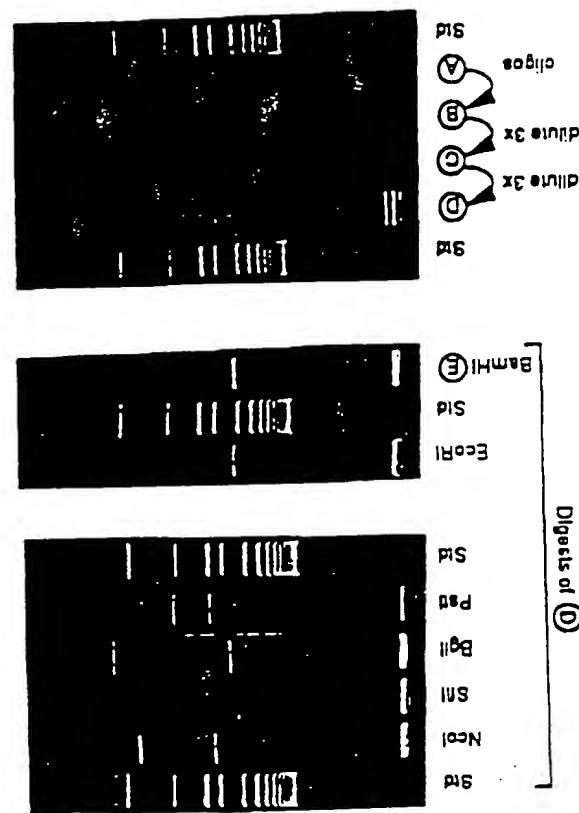
FIGURE 6

FIGURE 7

ALL 8 BP
ENZYMES:
NOT I
SFI I
ASC I
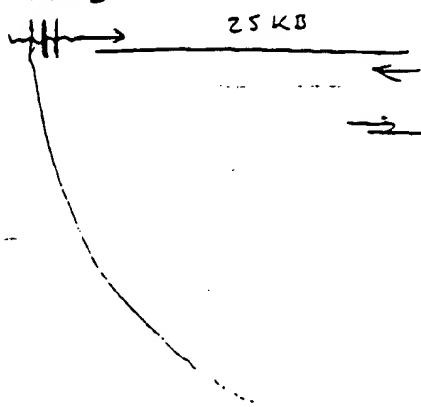
100 KB PCR

THIS CIRCULAR (SPIRAL) REACTION
COULD ALSO PERFORM VERY LONG RANGE PCR
AS OUTLINED BELOW: >100 KB?

(≡ PARALLELL PROCESSING)

25 KB

25 KB

28 KB

25 KB
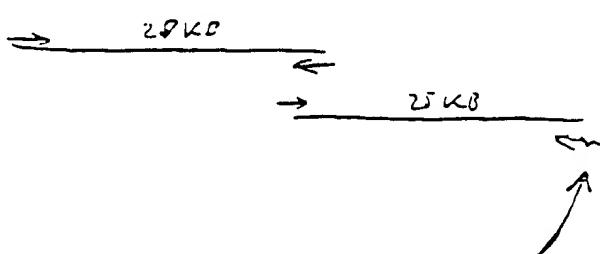
COLI CHROM. DNA: 4 SEPARATE REACTIONS (OR 1 RXN W. OVERLAPPING PRIMERS)

LINEARIZE W.:
NOT on
SFI on
ASC on
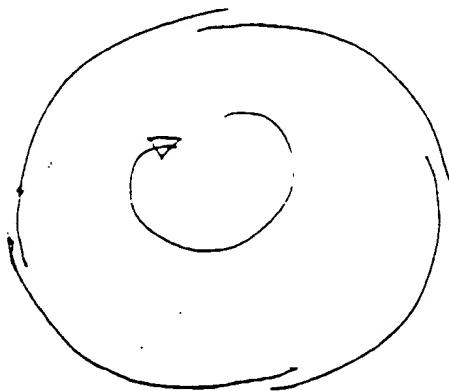
Figure 8

Continuous Circular Multiplex PCR

Initial Conditions

Anneal with Primers
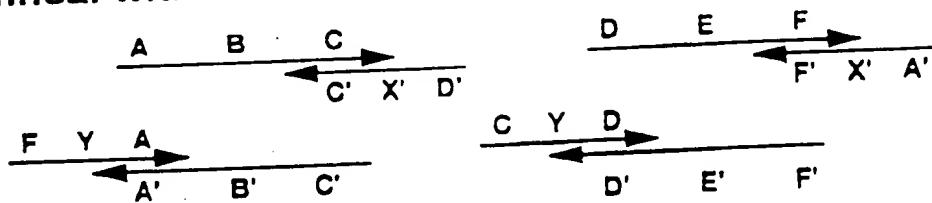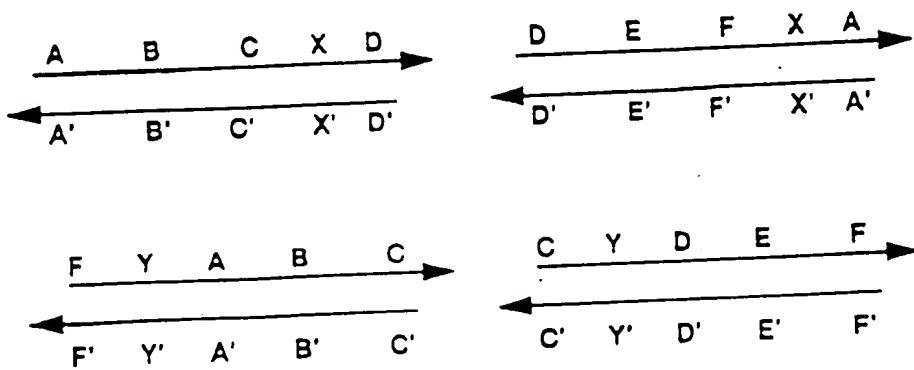
Extend and Melt

Figure 9A
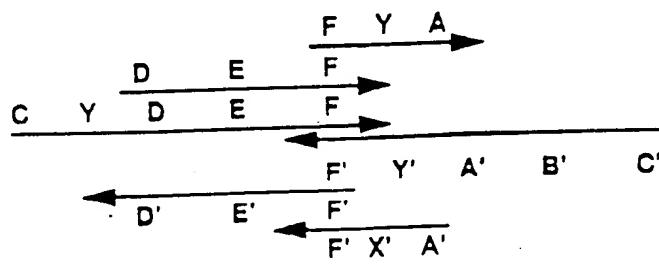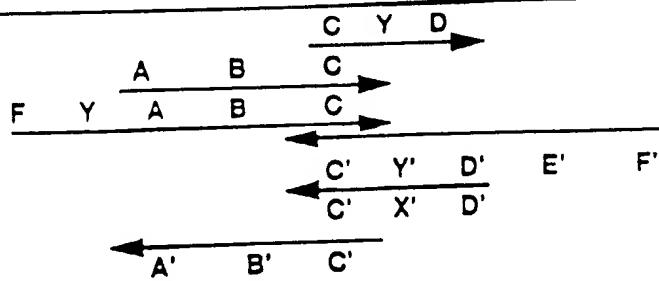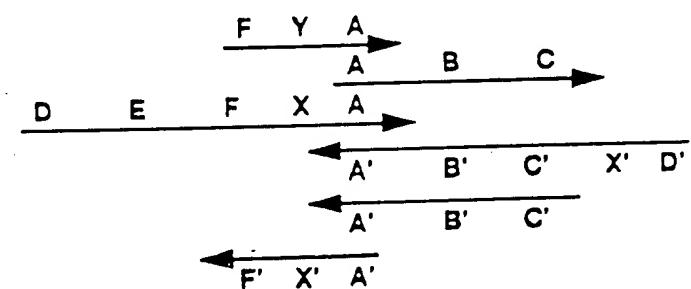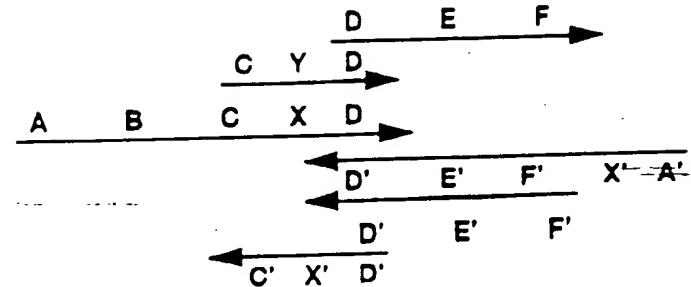
Reanneal

Figure 9B
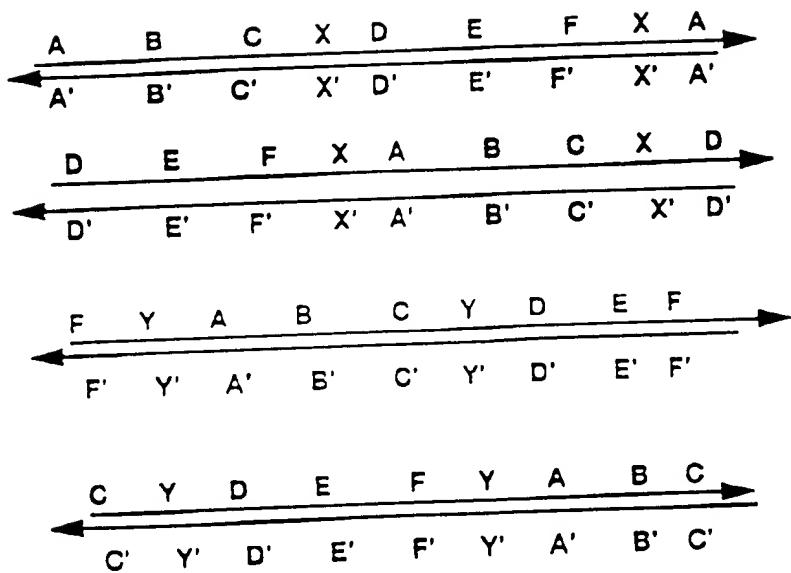
Extend

Figure 9C

Continuous Linear Multiplex PCR

Initial Conditions

Anneal with Primers
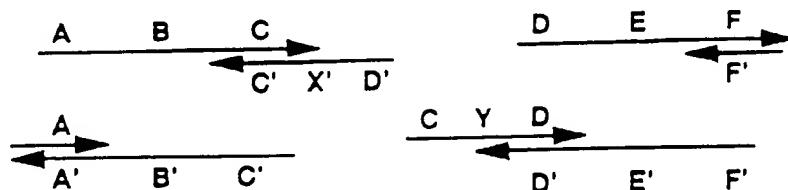
Extend and Melt
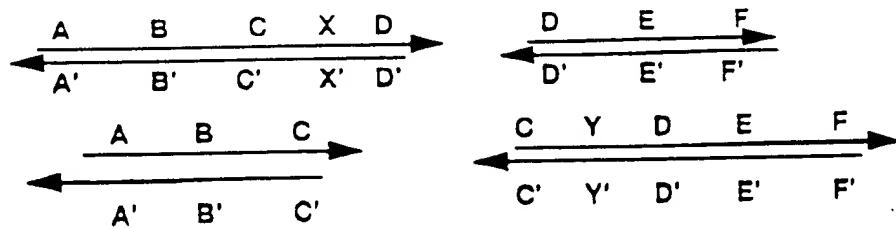
Figure 10A

Reanneal
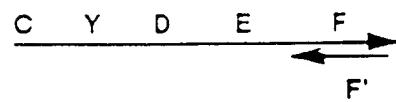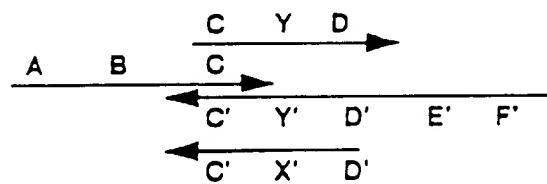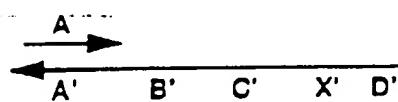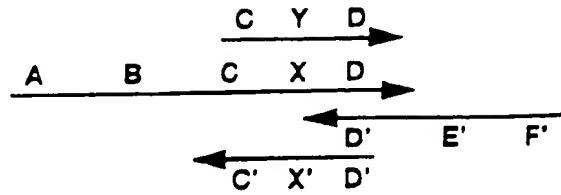
Figure 10B

Extend - After a period, assuming equimolar primers, only the following products are left.
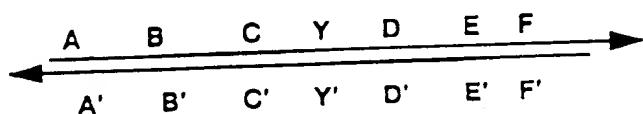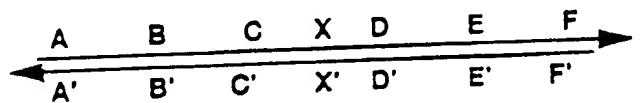
Amplify using only primers A and F '

If necessary, digest to separate the components using restriction sites incorporated into X and Y

Figure 10C

Initial Conditions: sequence embedded in genome DNA, indicated in gray
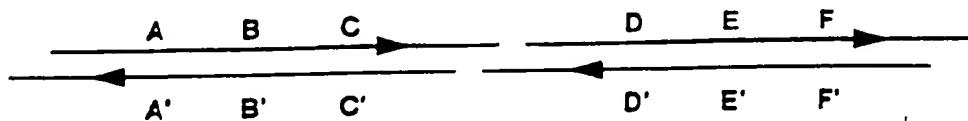
Anneal #1: Low concentration standard regular PCR Primers.
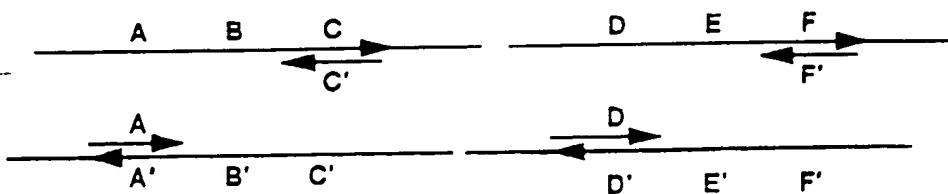
Figure 11A

Anneal with Primers: New primers are at higher concentration than initial primers so that there is no competition problem with these primers binding with the initial primers. Note that primers are selected for only one strand. The orientation is selected so that the resulting fragment overlap.
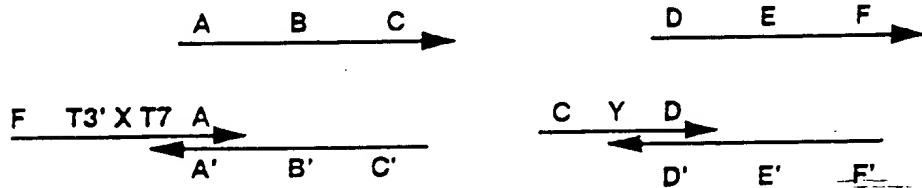

Extend and Melt:
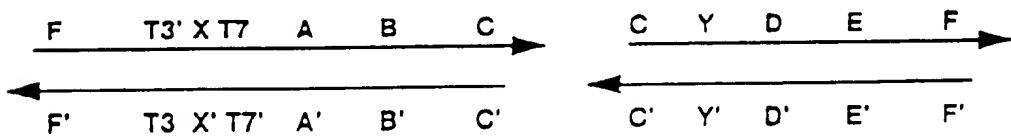
Figure 11 ⊆

Reanneal
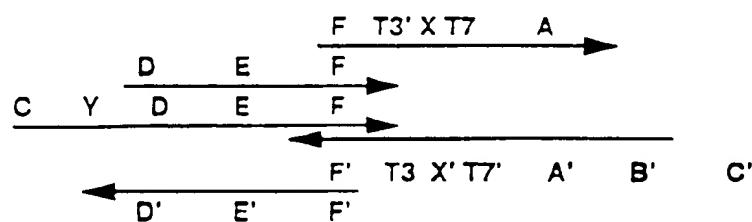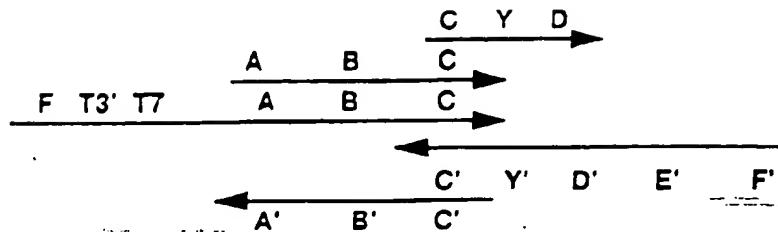
Extend
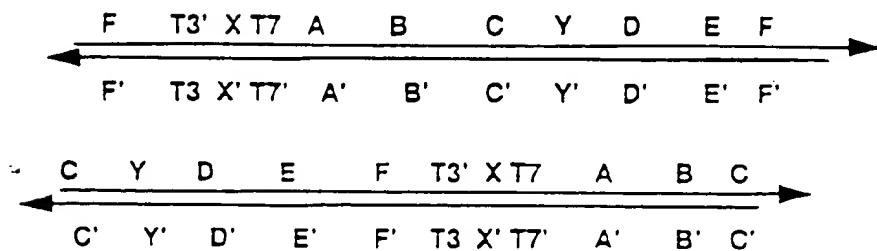
Figure 11C
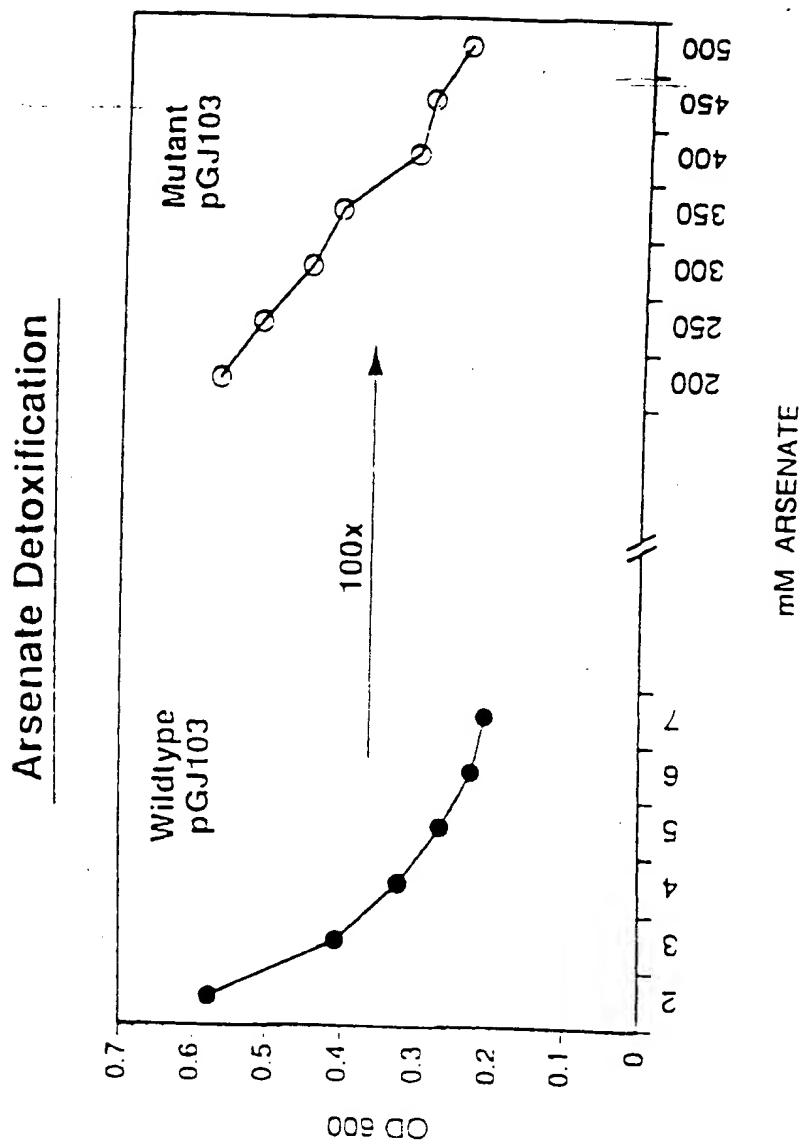
Figure 12
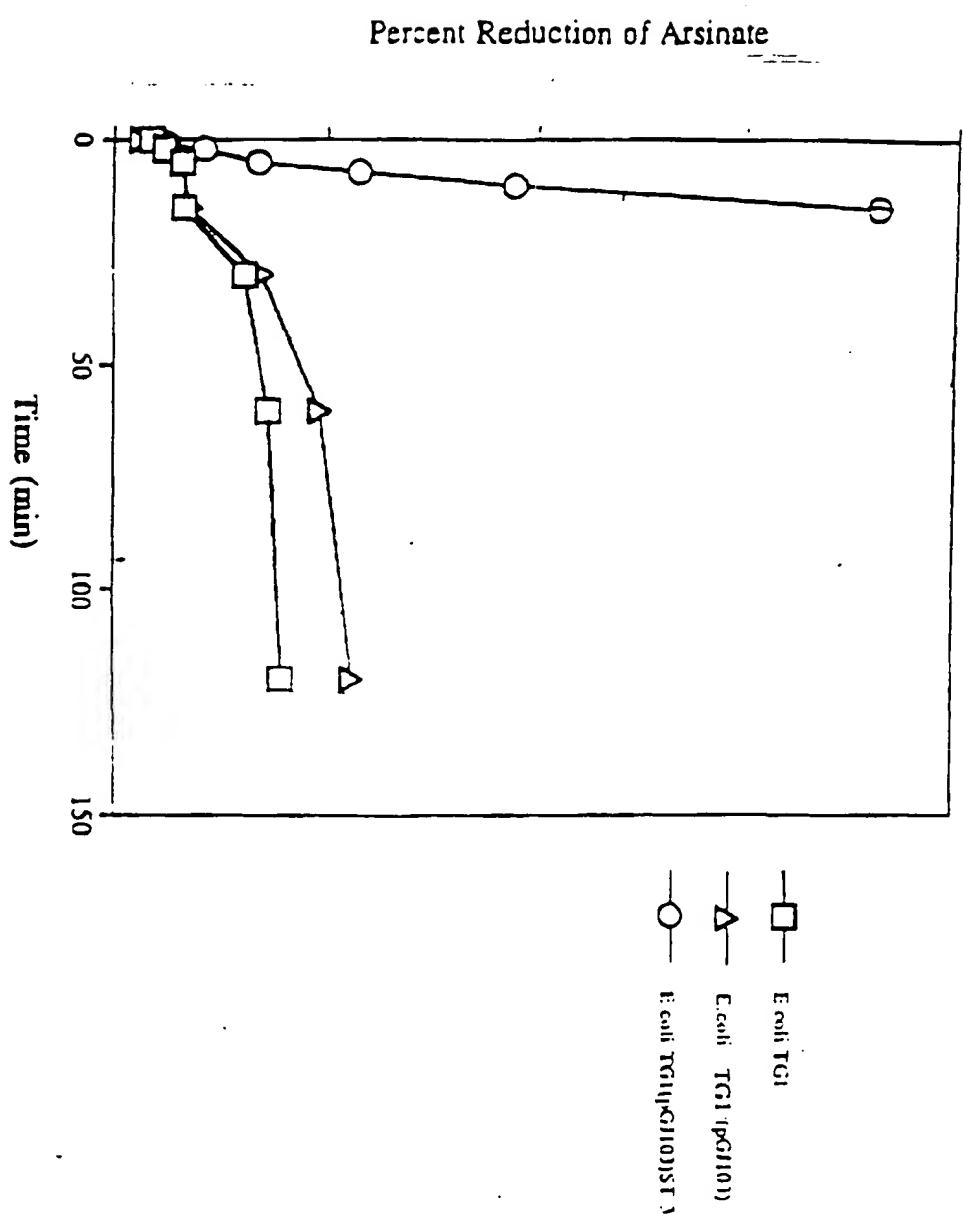
Figure 13

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07H 21/04
US CL   :536/24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   536/24.3; 435/91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, MEDLINE, BIOSIS,..CAPLUS
search terms: primers, PCR, head-tail , cycles, concatemer, ligation, amplification

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WHITE et al., Analytical Biochemistry, "Concatemer Chain Reaction: A Tag DNA Polymerase-Mediated Mechanism for Generating Long Tandemly Repetitive DNA Sequences", Volume 199, issued 1991, pages 184-190, especially pages 186-187.	1, 2, 5-12, 14-18 -------------- 3, 4, 13
Y	US 5,118,604 A (WEISMAN et al) 02 June 1992 , see column 11, lines 30-50.	3
Y	SUZUKI et al. In vivo ligation of linear DNA molecules to circular forms in the yeast saccharomyces cerevisiae. Journal of Bacteriology. August 1983, Vol. 155, No. 2, pages 747-754, especially pages 747, 750, 752.	4, 13

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents.	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 JULY 1996	19 SEP 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Joyce Tung
Facsimile No. (703) 305-3230	Telephone No (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*